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# Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal

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**Buck, Michael J., Teresa L. Squire, and Matthew T. Andrews.** Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. *Physiol Genomics* 8: 5–13, 2002. First published November 27, 2001; 10.1152/physiolgenomics.00076.2001.—Hibernation in mammals requires a metabolic shift away from the oxidation of carbohydrates and toward the combustion of stored fatty acids as the primary source of energy during torpor. A key element involved in this fuel selection is pyruvate dehydrogenase kinase isoenzyme 4 (PDK4). PDK4 inhibits pyruvate dehydrogenase and thus minimizes carbohydrate oxidation by preventing the flow of glycolytic products into the tricarboxylic acid cycle. This paper examines expression of the PDK4 gene during hibernation in heart, skeletal muscle, and white adipose tissue (WAT) of the 13-lined ground squirrel, *Spermophilus tridecemlineatus*. During hibernation PDK4 mRNA levels increase 5-fold in skeletal muscle and 15-fold in WAT compared with summer-active levels. Similarly, PDK4 protein is increased threefold in heart, fivefold in skeletal muscle, and eightfold in WAT. High levels of serum insulin, likely to have an inhibitory effect on PDK4 gene expression, are seen during fall when PDK4 mRNA levels are low. Coordinate upregulation of PDK4 in three distinct tissues suggests a common signal that regulates PDK4 expression and fuel selection during hibernation.

hibernation; pyruvate dehydrogenase kinase isoenzyme 4; insulin; peroxisome proliferator-activated receptor- $\alpha$ ; *Spermophilus tridecemlineatus*

PROFOUND CHANGES in body temperature, metabolism and heart rate allow hibernating mammals to survive seasonally cold environments for periods of several months with little or no food. To accomplish this feat, individual organ systems undergo metabolic rate depression, minimize the use of carbohydrates, and switch to stored triacylglycerols as their primary source of fuel (reviewed in Ref. 20). In the absence of feeding, conservation of glucose for utilization by the brain becomes essential for survival and is controlled by inhibition of the mitochondrial pyruvate dehydrogenase complex (23). In hibernating golden-mantled ground squirrels, Brooks and Storey (6) observed a 96%

reduction in cardiac pyruvate dehydrogenase activity during hibernation. In the hearts of Djungarian hamsters, Heldmaier et al. (16) showed that inactivation of pyruvate dehydrogenase closely correlates with metabolic rate reduction seen during daily torpor. Regulated long-term inhibition of pyruvate dehydrogenase activity is the result of phosphorylation of the pyruvate dehydrogenase complex by the activity of various isoenzymes of the pyruvate dehydrogenase kinase family (4).

In this paper we address the regulation of the gene encoding pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) in the hibernating 13-lined ground squirrel, *Spermophilus tridecemlineatus*. We previously identified PDK4 as an upregulated gene in the ground squirrel heart during hibernation (1). Inhibition of pyruvate dehydrogenase by PDK4-mediated phosphorylation reduces glucose oxidation by preventing the conversion of pyruvate to acetyl-CoA and CO<sub>2</sub>, thus stopping the flow of glycolytic intermediates into the tricarboxylic acid (TCA) cycle (24). PDK4 mRNA exhibits its highest levels of expression in heart and skeletal muscle in both humans (24) and rats (4). More moderate levels are also seen in brain, placenta, lung, liver, kidney, and pancreas in humans (24) and in lung, liver, and kidney in rats (4). Among the PDK isoenzymes, expression of PDK4 appears to be the most responsive to physiological change (reviewed in Ref. 15). Starvation (30–33) and diabetes (31, 33) are two conditions that stimulate PDK4 expression and show physiological characteristics related to the hibernating state.

Here we report on PDK4 gene expression in 10 different ground squirrel tissues and quantify mRNA and protein levels in those tissues showing the highest levels of expression: heart, white adipose tissue (WAT), and skeletal muscle from the thigh (quadriceps femoris). Our analysis begins in late summer and continues through the hibernation season to spring arousal when the animal resumes its activities. We also explore the regulation of PDK4 by examining the seasonal expression of insulin. Insulin is a key metabolic hormone that stimulates glucose uptake from the blood, activates glycogen synthase, inactivates glycogen breakdown, and stimulates the storage of excess fuel as fat. In diabetic rats an increase in insulin concentration has been shown to reduce the levels of both PDK4 protein and mRNA in heart and skeletal muscle (31, 33). We have measured insulin levels during hibernation,

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cloned the ground squirrel insulin cDNA, and measured insulin gene expression in the pancreas throughout the hibernation season. These determinations have allowed us to contrast levels of PDK4 mRNA and protein with insulin mRNA and protein at various times of the year such as summer when the heartbeat is rapid (200–300 beats/min) and legs are flexing, vs. deep hibernation when the heart rate slows (2–10 beats/min) and the legs are inactive. Based on the results described in this paper and on other recent studies on PDK4 regulation, we propose a regulatory mechanism that accounts for the switch from carbohydrate to fatty acids as the primary source of fuel during hibernation.

## MATERIALS AND METHODS

**Animals.** Animal care and use was in accordance with Institutional Animal Care and Use Committee guidelines. Thirteen-lined ground squirrels (*S. tridecemlineatus*) were received from TLS Research (Bartlett, IL) within 3–4 days of wild capture during the first week of August. Active August animals were maintained with water and killed within 24 h of arrival from the supplier. All other animals were maintained in captivity on a diet of standard rodent chow supplemented with sunflower seeds and water ad libitum. All squirrels were maintained at 23°C in August, 17°C in September, 11°C in October, and 5°C from November through mid-March. Animals were housed with a 12:12-h light/dark cycle from August through the end of October. From November to mid-March the animals were housed in total darkness with only water ad libitum. Squirrels were observed daily during these months and hibernation patterns were determined using the sawdust technique (22). Hibernating animals were at least in *day 2* of a torpor bout when killed, and animals in interbout arousal (IBA) were collected after at least three previous hibernation bouts of 8 days or more. IBAs are naturally occurring interruptions of the dormant state when hibernating animals raise their body temperatures and show brief periods of activity (29). The time of death within an IBA was not determined. Hibernating animals killed in October were often in their first torpor bout of the season, whereas hibernating animals collected from December through early March had experienced at least three previous torpor bouts of 8 days or more. The hibernation phase of the study was concluded in mid-March by increasing room temperature to 23°C, re-establishing 12:12-h dark/light cycle, and providing a diet of standard rodent chow and sunflower seeds ad libitum. Rectal temperatures were measured at the time of death. Ground squirrel tissue was promptly removed and frozen immediately in liquid nitrogen. Pancreatic tissue was immersed directly in ice-cold guanidinium isothiocyanate, and RNA was prepared immediately because of high levels of RNase activity in the pancreas. Whole blood was stored on ice, serum was prepared promptly, distributed into aliquots, frozen in liquid nitrogen, and stored at –80°C until time of assay.

**RNA isolation.** Total RNA was prepared from ground squirrel tissues using the method described by Andrews et al. (1) or by a modification of the protocol provided with the Totally RNA kit from Ambion. For the latter method, all solutions were from the Totally RNA kit. The tissue was homogenized with a rotary-blade homogenizer in guanidinium isothiocyanate. The WAT homogenate was subjected to an initial centrifugation for 10 min at 3,000 *g*, after which the glycerol and lipid layer was removed. The homogenate for

all tissues was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by addition of sodium acetate to a final concentration of 0.2 M. This mixture was extracted once more with acid-phenol:chloroform (1:1). All extractions were performed using phase-lock tubes (Eppendorf) at 4,500 *g* in a swinging bucket rotor. The aqueous layer was divided into aliquots and stored as isopropanol precipitates. Individual aliquots were centrifuged, rinsed with 70% ethanol, dissolved in water, and quantified by absorption spectrophotometry.

**Northern blots.** Total RNAs from designated tissues of active and hibernating 13-lined ground squirrels were separated on agarose gels containing formaldehyde followed by transfer onto MagnaCharge nylon membranes (Osmonics) using 10× SSPE according to Sambrook et al. (25). The multi-tissue blot used to examine PDK4 mRNA levels in 10 different hibernating tissues utilized 15 µg total RNA per lane. Individual heart, WAT, and skeletal muscle blots, used to examine PDK4 mRNA levels at various time points throughout the hibernation season, utilized 8 µg total RNA per lane. These Northern blots were hybridized with a random-primed (8, 9) <sup>32</sup>P-labeled PDK4 cDNA (accession no. AF020845). Pancreas Northern blots used for detecting insulin mRNA contained 10 µg of total RNA per lane. These blots were hybridized with a <sup>32</sup>P-end-labeled (25) insulin oligonucleotide (5′ TTGCAGTAGTTCTCCAGCTGGTAGAGGGAGCAGATG 3′).

RNA integrity was examined using a 1% agarose, 1× TAE non-denaturing gel stained with ethidium bromide. Loading and integrity of RNA on Northern blots were examined by hybridization with a <sup>32</sup>P-end-labeled (25) 18S rRNA oligonucleotide (5′ CGACTTTTACTTCCTCTAGATAGTCAAGTTCGAC 3′). Oligonucleotide sequences for insulin and 18S rRNA were constructed from conserved domains for each gene product as determined by multi-sequence alignment with several mammalian homologs. PDK4 and insulin mRNA levels were determined by a STORM phosphorimager using ImageQuant software (Molecular Dynamics).

**Library construction.** A pancreas cDNA library was constructed from pancreatic poly(A)<sup>+</sup> mRNA isolated from both a hibernating and an active 13-lined ground squirrel. A directional cDNA library was created using the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (GIBCO-BRL, Life Technologies) according to the manufacturer's protocol. The library was screened for the insulin cDNA using the <sup>32</sup>P-end-labeled insulin oligonucleotide described in the *Northern blots*, above.

**Serum insulin assay.** Whole blood collected at death was spun at 3,500 *g* for 10 min, and partially purified serum was transferred to 1.5-ml tubes and spun at 3,500 *g* for 15 min at 4°C. Serum was divided into aliquots, frozen in liquid nitrogen, and stored at –80°C until time of assay. Serum was thawed on ice, and the insulin concentration was determined by radioimmunoassay (rat insulin RIA kit, Linco). The minimal detection limit of the serum insulin assay is 0.1 ng/ml. Two different concentrations of purified rat insulin provided by the manufacturer served as quality controls. We measured the concentration and standard deviation of the control samples by replication before beginning the assays. Experimental assays contained ground squirrel serum samples and the quality controls. In this manner we assured ourselves that any assay is repeatable, because both of the quality control values were within two standard deviations. Samples from the same animals were performed in duplicate, and the difference between the duplicates was less than 10%.

**Western blots.** Protein from WAT, heart, and skeletal muscle (quadriceps femoris) was prepared as acetone powder (14). Powders were solubilized in 0.2 M Tris·HCl (pH 9.2) and

1 M ethylene glycol and reconstituted by gentle agitation for 2–3 h at 4°C. The total solubilized protein concentration was determined by Bradford assay using dye reagents from Bio-Rad laboratories. Ten micrograms of solubilized protein was boiled in loading buffer (1.5 M Tris·HCl pH 8.9, 1.2% SDS, 1% DTT) and separated on the basis of size on a 10% polyacrylamide gel by discontinuous SDS-PAGE. The separated proteins were subsequently transferred electrophoretically to NitroBind nitrocellulose membrane (Osmonics). Membranes were then blocked overnight at room temperature with TBST (75 mM NaCl, 10 mM Tris·HCl, 0.5% Tween 20, pH 9.2) augmented with 3% (wt/vol) bovine serum albumin. For detection, membranes were incubated for 2 h with a 1:10,000 dilution of polyclonal antisera against rat PDK4 (a gift from R. A. Harris). After three 10-min washes with TBST, membranes were incubated with anti-rabbit IgG secondary antibody linked with horseradish peroxidase (1:5,000, in TBST) for 1 h. The blots were given four 10-min washes in TBST, and bound antibody was visualized using the ECL detection system (Amersham). The blots were exposed to film, quantified by scanning densitometry, and analyzed with ImageQuant software (Molecular Dynamics). Films used for scanning densitometry were not overexposed to assure that all densitometric quantifications were within the linear range of the film. Exposures used for densitometry involved 56 different tissue samples (heart, 19 animals; skeletal muscle, 18 animals; WAT, 19 animals) quantified on films different from those shown for demonstration purposes in Fig. 3.

**Statistical analysis.** For Northern blots, loading was normalized by 18S rRNA, and relative values were determined by comparison to the average August value. For Western blots, an equal amount of soluble protein was loaded for each sample, and relative PDK4 protein levels were then determined by comparison to the average August amount. For serum insulin levels, absolute concentrations were used for statistical comparisons. One-way ANOVAs were used to determine significant differences in levels when compared with August active values. The ANOVA calculations were performed using version 7.1 of the SAS system (SAS Institute, Cary, NC).

## RESULTS

During hibernation in the 13-lined ground squirrel (*S. tridecemlineatus*) PDK4 mRNA was seen at relatively low levels in the adrenals, brain stem, cerebrum, kidney, liver, pancreas, and testes; and at much higher levels in heart (1), skeletal muscle, and WAT (Fig. 1A). PDK4 mRNA was also detected during hibernation in the heart of the golden-mantled ground squirrel (*S. lateralis*) and in arctic ground squirrel (*S. parryi*) heart and WAT (data not shown). Our investigation of PDK4 expression in 13-lined ground squirrels was conducted throughout the hibernation season to determine whether there was evidence for common regulation among the three tissues showing the highest mRNA levels. Total RNA from heart, skeletal muscle from the thigh (quadriceps femoris), and abdominal WAT were prepared from individual animals at various states of activity from August through March.

Expression of the PDK4 gene was similar in heart, skeletal muscle and WAT throughout the year (Fig. 1B). PDK4 mRNA levels were relatively unchanged until the animal entered hibernation in October. Hibernating animals in October showed a slight nonsig-

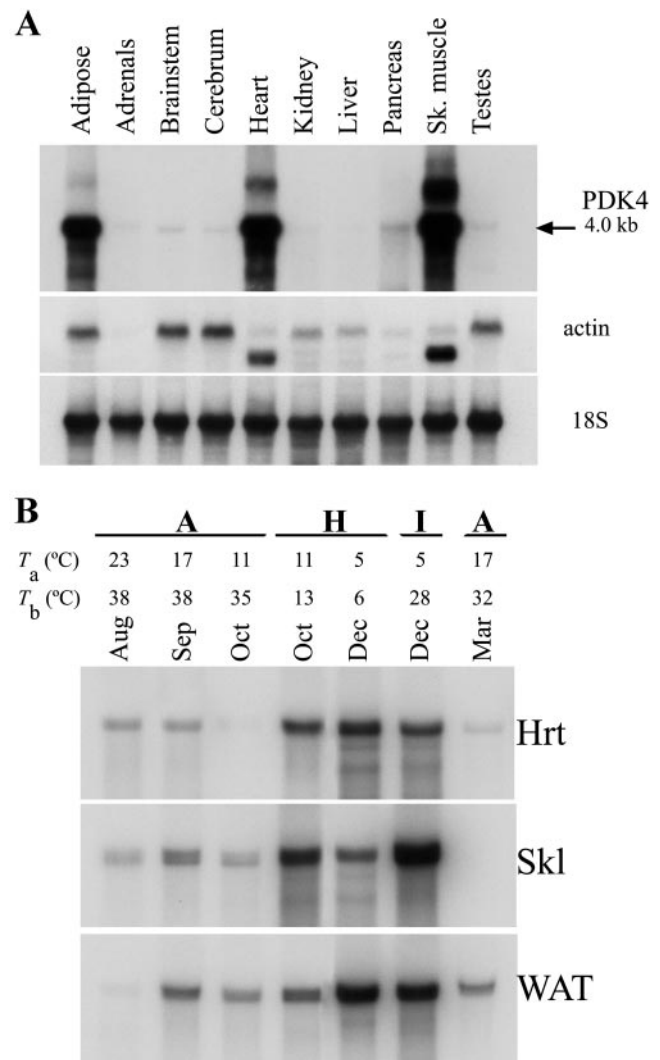


Fig. 1. Pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) gene expression in 13-lined ground squirrels. **A:** Northern blot containing total RNA (15  $\mu$ g/lane) isolated from multiple ground squirrel tissues during hibernation. Tissues were taken from multiple animals killed in December and January with body temperatures ranging from 6 to 10°C. Tissues are indicated above each lane, and the  $\beta$ -actin mRNA ("actin") and 18S rRNA ("18S") profiles are shown directly below. **B:** Northern blot of total RNA (8  $\mu$ g/lane) from heart (Hrt), skeletal muscle (SkI), and white adipose tissue (WAT) of the same animal for each time point. Ambient temperature ( $T_a$ ), body temperature ( $T_b$ ), and month of death are indicated above each lane. The state of each animal is indicated as active (A), hibernating (H), or in interbout arousal (I). RNA integrity and gel loading was controlled by hybridization with 18S rRNA (not shown).

nificant increase in expression in all three tissues relative to August values as shown graphically for skeletal muscle (Fig. 2A) and WAT (Fig. 2B). Later in the hibernation season (beginning in December) there was a further increase in mRNA levels, with the greatest increase relative to August values seen during IBAs. After hibernation ceased in the spring, PDK4 message dropped back to near August levels. In skeletal muscle, PDK4 message was increased 5-fold during hibernation and 7-fold during IBAs, compared with August active values ( $P < 0.01$ ; Fig. 2A). In WAT, there

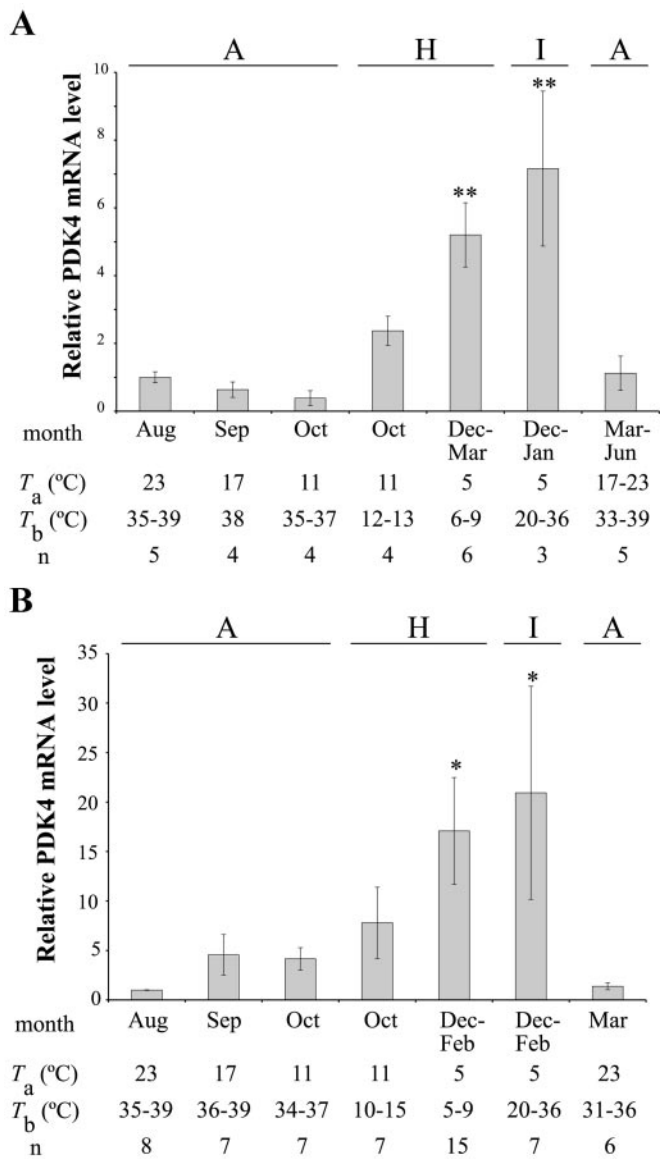


Fig. 2. Summaries of PDK4 mRNA levels in skeletal muscle (A) and WAT (B). Similar measurements of PDK4 mRNA levels in heart are published (1). Ambient temperature ( $T_a$ ), body temperature ( $T_b$ ), and month(s) of death are indicated below each bar. The state of each animal is indicated above as active (A), hibernating (H), or in interbout arousal (I). The PDK4 level was normalized to the average August level. The number of animals ( $n$ ) for each time point is indicated. Statistically significant comparisons to August mRNA levels were determined by one-way ANOVA: \* $0.01 < P < 0.05$  and \*\* $P < 0.01$ .

was a 15-fold increase in PDK4 mRNA during hibernation and a 20-fold increase during IBAs ( $P < 0.05$ ; Fig. 2B). Relative levels of PDK4 mRNA in heart were quantified previously (1) and closely resemble those seen in WAT.

Western blot analysis using antiserum against rat PDK4 (a gift from R. A. Harris) was employed to measure 13-lined ground squirrel PDK4 protein levels in heart, skeletal muscle, and WAT (Fig. 3). PDK4 protein levels in these three tissues were consistent with their mRNA profiles. In active August animals,

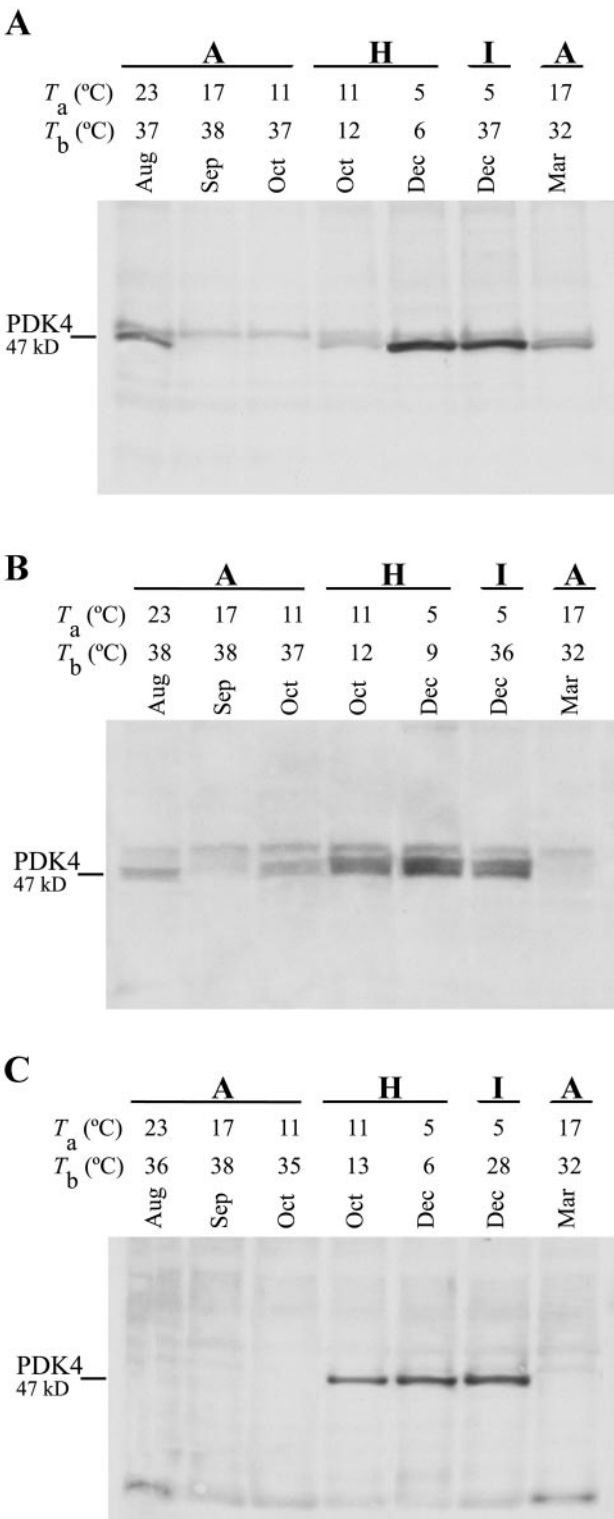


Fig. 3. Western blots of total protein (10  $\mu$ g/lane) isolated from heart (A), skeletal muscle (B), and WAT (C). The blots were probed with anti-rat PDK4 antiserum. Ambient temperature ( $T_a$ ), body temperature ( $T_b$ ), and month of death are indicated above each lane. The state of each animal is indicated as active (A), hibernating (H), or in interbout arousal (I). The size of PDK4 in kilodaltons (kDa) is indicated on the left of each blot.

PDK4 protein was found at low levels within heart and skeletal muscle but was barely detectable in WAT. Compared with August squirrels, PDK4 protein declined significantly in hearts from active September-October animals ( $P < 0.01$ ) and then increased threefold in hibernating hearts ( $P < 0.01$ ; Fig. 4A). In skeletal muscle, PDK4 protein remained relatively unchanged from August until entrance into hibernation, when its level increased fivefold ( $P < 0.05$ ) and reached its maximum during IBAs ( $P < 0.01$ ; Fig. 4B). In WAT, PDK4 protein increased twofold ( $P < 0.05$ ) in September-October animals and eightfold (nonsignificant) in hibernating animals (Fig. 4C). When comparing hibernating and nonhibernating animals killed on the same day in October, the hibernating animal has an elevated

level of PDK4 protein in all three tissues (Fig. 3). This difference was most evident in WAT in which the PDK4 protein, barely detectable during summer and fall, was seen at high levels after animals entered hibernation in October and then remained high during hibernation (Fig. 3C). After hibernation concluded in spring, the relative concentration of PDK4 protein dropped to summer levels in all three tissues examined (Figs. 3 and 4).

The coordinate expression of PDK4 in different tissues during hibernation prompted us to measure circulating insulin levels because of the long-standing observation that insulin has a controlling influence on pyruvate dehydrogenase kinase activity (17). More recent experiments with rat hearts (33) and skeletal

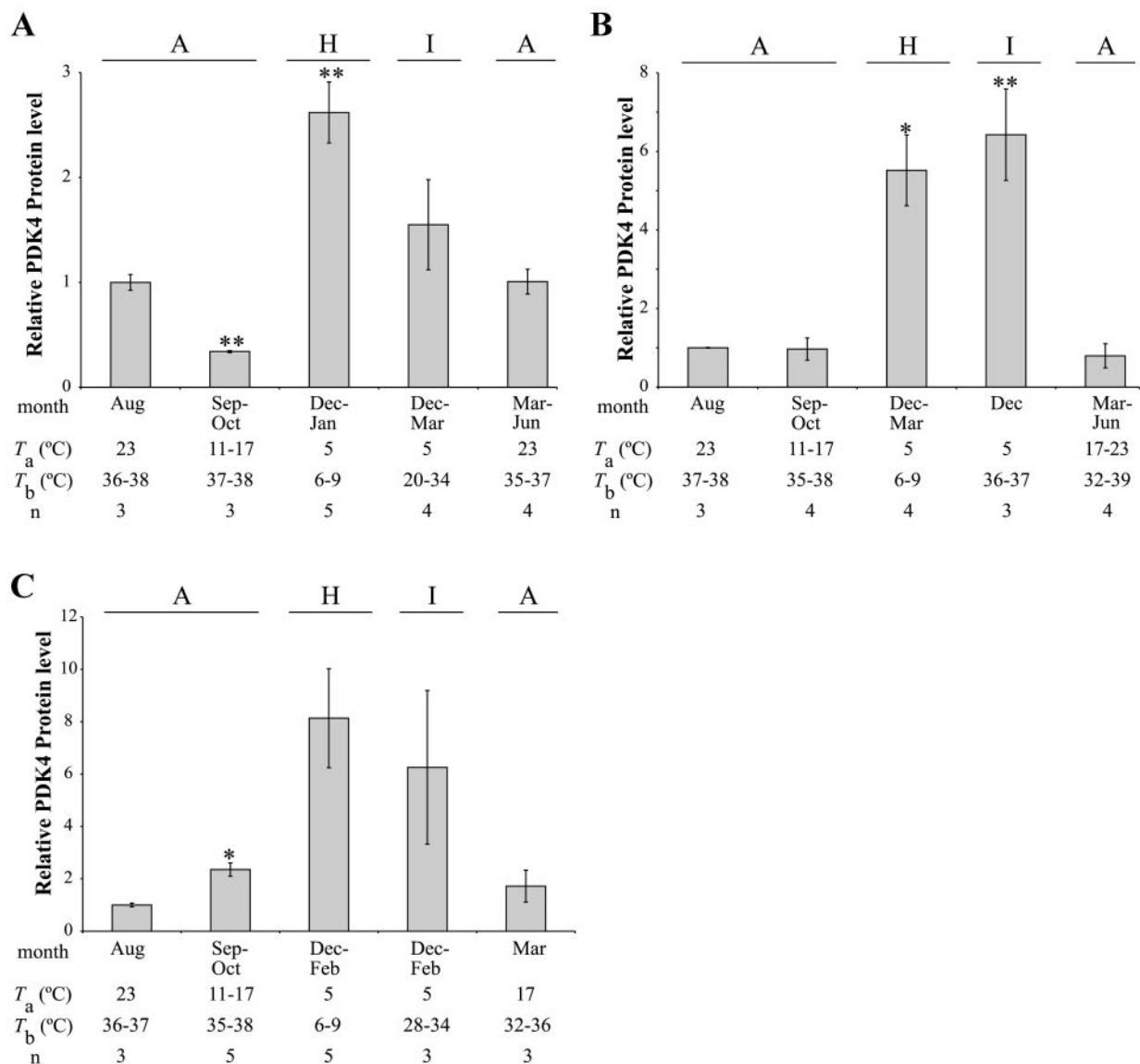


Fig. 4. Summaries of PDK4 protein levels in heart (A), skeletal muscle (B), and WAT (C). Ambient temperature ( $T_a$ ), body temperature ( $T_b$ ), and month(s) of death are indicated below each bar. The activity state is indicated above as active (A), hibernating (H), or in interbout arousal (I). The number of animals ( $n$ ) for each time point is indicated. The PDK4 level was normalized to the average August value. Statistically significant comparisons to August protein levels were determined by one-way ANOVA: \* $0.01 < P < 0.05$  and \*\* $P < 0.01$ .

muscle (31) have shown high levels of PDK4 mRNA were induced in animals rendered diabetic by treatment with an antibiotic that destroys the insulin-producing capacity of the pancreas. Administration of insulin to these diabetic rats lowered PDK4 mRNA back

to control levels. In ground squirrels, we found the serum insulin concentration below 1 ng/ml for most of the year except during September and October, when the insulin concentration was increased fourfold ( $P < 0.01$ ) compared with August active animals (Fig. 5A).

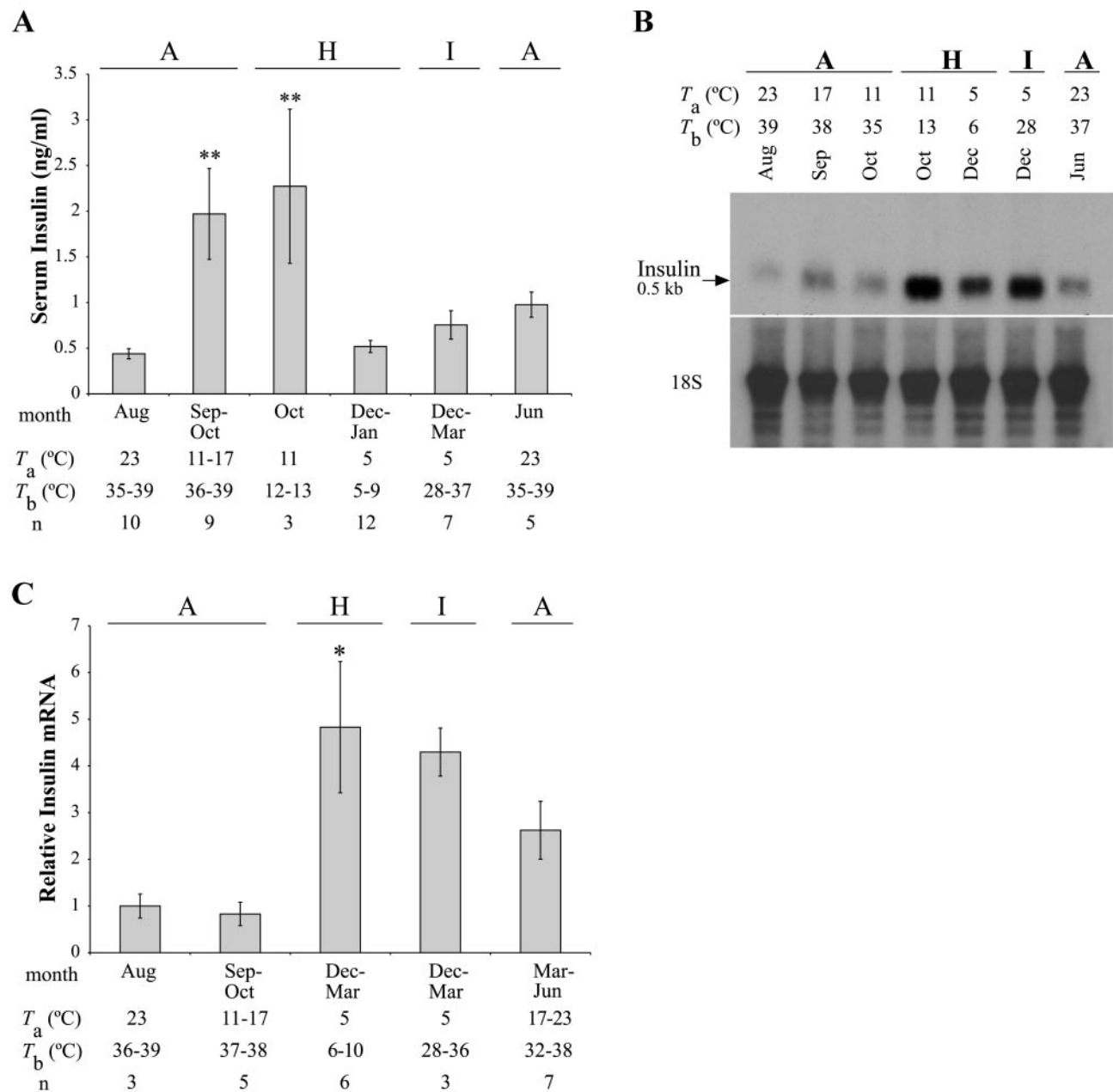


Fig. 5. Seasonal expression of insulin. A: serum insulin levels at various times of the year. Insulin concentration was determined by radioimmunoassay. Ambient temperature ( $T_a$ ), body temperature ( $T_b$ ), and month(s) of death are indicated below each bar. The activity state is indicated above as active (A), hibernating (H), or in interbout arousal (I). The number of animals ( $n$ ) for each time point is indicated. Statistically significant comparisons to August serum insulin levels were determined by one-way ANOVA:  $**P < 0.01$ . B: insulin gene expression in pancreas. Northern blot of total RNA (10  $\mu$ g per lane) isolated from pancreas and probed with an insulin-specific oligonucleotide. Ambient temperature ( $T_a$ ), body temperature ( $T_b$ ), and month of death are indicated above each lane. The state of each animal is indicated as active (A), hibernating (H), or in interbout arousal (I). The 18S rRNA (18S) profile in the pancreas is shown directly below. The size of the insulin message is indicated in kilobases (kb). C: summary of relative insulin mRNA level in the pancreas. The insulin mRNA level was normalized to the average August level. The number of animals ( $n$ ) for each time point is indicated. Statistically significant comparisons to August mRNA levels were determined by one-way ANOVA:  $*0.01 < P < 0.05$ .

When serum insulin is high in September-October, active animals show significantly lower PDK4 protein levels in heart compared with August animals, and all three tissues show lower PDK4 levels than hibernating animals (Fig. 4).

A full-length insulin cDNA (accession no. AY038604) was isolated from a 13-lined ground squirrel pancreatic cDNA library derived from hibernating and nonhibernating animals. Ground squirrel preproinsulin is 110 amino acids long and 89% identical to the unprocessed human protein (accession no. P01308). To investigate insulin gene expression, total RNA from pancreas was prepared from individual animals at various states of activity from August through June, immobilized on Northern blots, and probed with an end-labeled oligonucleotide complementary to mammalian insulin mRNA (Fig. 5B). Examination of insulin expression in the pancreas across the hibernation season revealed that mRNA levels within the pancreas did not correlate with serum insulin concentrations. When serum insulin levels were near their lowest point in December-January hibernating animals (Fig. 5A), insulin mRNA was upregulated fourfold ( $P < 0.05$ ) compared with summer active animals (Fig. 5C). Elevated levels of insulin mRNA in the pancreas during hibernation may be held in reserve so that insulin protein can be released when needed such as resumption of feeding and subsequent glucose utilization at spring arousal.

## DISCUSSION

During hibernation, carbohydrates are spared and fatty acids become the primary source of fuel as observed by a respiratory quotient (RQ) of 0.7 (reviewed in Ref. 20). RQ is a unitless value representing the moles of  $\text{CO}_2$  respired per moles of  $\text{O}_2$  consumed. A value of 1.0 indicates combustion of carbohydrates; however, an RQ of 0.7 indicates that fat is the major substrate for energy production. The enzyme PDK4

inhibits carbohydrate oxidation by phosphorylating the pyruvate dehydrogenase complex, thus impairing the flow of glycolytic intermediates into the TCA cycle. We found that the gene encoding PDK4 is upregulated during hibernation in heart, WAT, and skeletal muscle of the 13-lined ground squirrel, *S. tridecemlineatus*. Hibernation-associated expression of the PDK4 gene results in increased mRNA and protein levels in all three tissues.

Identification of specific genes associated with the hibernating state allows researchers to identify and test endogenous and exogenous factors that control differential gene expression during hibernation. Factors important in hibernation-related gene activity may be environmental such as cooler ambient temperatures and fewer daylight hours; and/or internal factors such as circannual rhythms and changes in metabolites and hormone levels. Coordinate activation of the gene encoding PDK4 in different tissues throughout the body points to circulating effector molecules that regulate its expression during hibernation. We propose a model (Fig. 6) showing how changes in serum insulin and fatty acid levels regulate PDK4 expression and ultimately the switch from carbohydrate- to fat-based catabolism during hibernation. Figure 6 extends our previous model (27) on the regulation of genes controlling metabolism during hibernation and is based on results reported in this paper and recent experimental findings described in the following paragraphs.

Injection of insulin into diabetic rats has been shown to reduce the levels of both PDK4 protein and mRNA in heart and skeletal muscle (31, 33). In 13-lined ground squirrels we found high levels of serum insulin in fall active animals coincide with low levels of PDK4 expression in heart, skeletal muscle, and WAT. An exception is seen in hibernating October animals when the level of PDK4 mRNA increases while the concentration of

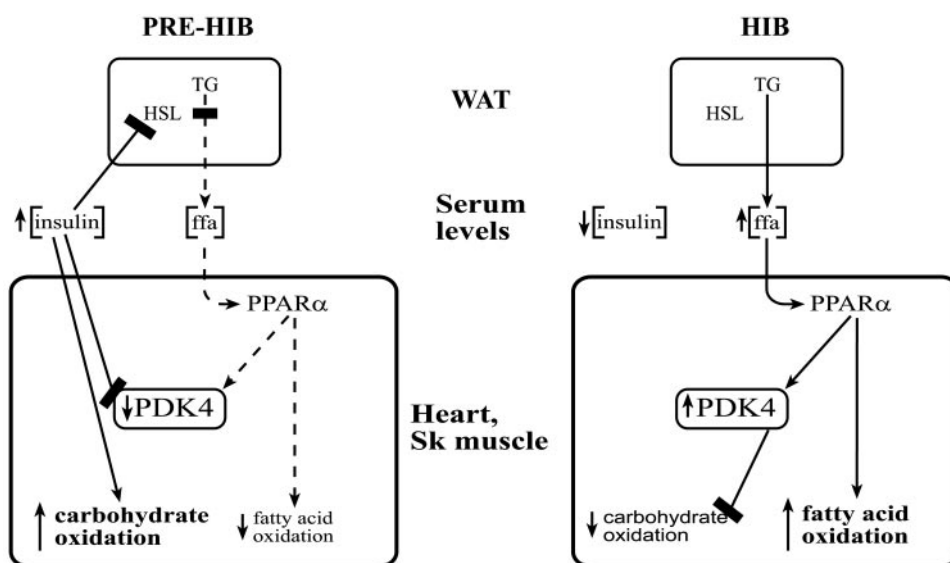


Fig. 6. Model showing regulation of PDK4 gene expression and the switch from carbohydrate to fatty acids as the primary source of fuel during hibernation: effects of serum levels of insulin and free fatty acids (ffa) on PDK4 expression, carbohydrate oxidation, and fatty acid oxidation in heart and skeletal muscle of active (PRE-HIB; September-October) and hibernating (HIB; December-January) animals. Long lines with arrowheads indicate upregulation or activation, and lines with blunt ends indicate downregulation or inhibition. Long solid lines show the predominant mode of regulation, and dashed lines show potential secondary regulation. Short vertical arrows pointing up indicate an increase in concentration or activity. Short vertical arrows pointing down indicate a decrease in concentration or activity. HSL, hormone-sensitive lipase; PPAR $\alpha$ , peroxisome proliferator activated receptor- $\alpha$ ; TG, triacylglycerols.

serum insulin remains high. Despite the lack of feeding, high serum insulin levels in October persist for the first few days after hibernation begins and may be the result of reduced insulin turnover due to low body temperatures (12–13°C). The increase in PDK4 mRNA is probably due to a competing PDK4-activation pathway (Fig. 6; described below) and/or by the onset of insulin resistance like that observed in another hibernator, the yellow-bellied marmot (*Marmota flaviventris*; Ref. 10). Insulin resistance is a reduction in the ability of insulin to regulate glucose homeostasis, causing an increase in serum insulin or hyperinsulinemia. Florant and colleagues (10) suggest that hyperinsulinemia and peripheral insulin resistance are maximal as the animal ceases to feed and enters hibernation.

Our observation that levels of serum insulin increase in active fall animals (September–October) and then decline to less than 1 ng/ml in hibernating December–January animals is also seen in the yellow-bellied marmot (28). Seasonal insulin concentrations in the marmot are similar to those shown in Fig. 5A for 13-lined ground squirrels. Despite the depressed serum insulin levels in December–January, we found that insulin mRNA levels in the pancreas are the highest in hibernating animals collected December through March (Fig. 5C). This observation is analogous to high insulin protein levels retained in the pancreas of the hibernating little brown bat (*Myotis lucifugus*; 3). During hibernation the level of bat insulin protein steadily increases in the pancreas, reaching its maximal level just prior to spring arousal. Bauman (3) notes that the increase in pancreatic insulin concentration, from early to late hibernation, may provide a rapidly releasable storage pool required for immediate secretion during spring arousal. This suggestion could explain the increase in insulin mRNA that we report in hibernating 13-lined ground squirrels (Fig. 5, B and C).

While insulin acts as an inhibitor of PDK4 expression, a member of the peroxisome proliferator-activated receptor (PPAR) family is known to activate the PDK4 gene. PPARs are ligand-dependent nuclear receptors that heterodimerize with the retinoid X receptor (19, 21) and thereby act as transcription factors. PDK4 expression is induced in skeletal muscle of rats and the hearts of mice by a hypolipidemic drug and known peroxisome proliferator, WY-14,643 (31, 32). This finding indicates a role for PPAR $\alpha$  in controlling PDK4 gene expression due to the fact that WY-14,643 is a selective PPAR $\alpha$ -activating ligand (12). This role of PPAR $\alpha$  in activating the PDK4 gene has also recently been confirmed using PPAR $\alpha$ -null mice (32).

Natural PPAR-activating ligands include long-chain free fatty acids, such as linoleic acid, linolenic acid, and arachidonic acid (18). Elevated levels of fatty acids are observed in serum both before and during hibernation (11, 13). This elevation is likely a consequence of increased food intake in the months leading up to hibernation when the animal is fattening and preparing for dormancy. In 13-lined ground squirrels, elevated serum fatty acid levels can also result from pancreatic triacylglycerol lipase-mediated lipolysis of triacylgly-

cerols stored in WAT (2). Unlike hormone-sensitive lipase, which is the primary lipolytic enzyme in WAT, pancreatic triacylglycerol lipase is not inhibited by insulin and can therefore provide a steady supply of free fatty acids before and during hibernation. The suggestion that free fatty acids can activate PDK4 expression in the presence of insulin is strengthened by a recent line of evidence showing that insulin is not effective in opposing or reversing WY-14,643 activation of the PDK4 gene in Morris 7800C1 hepatoma cells (15). This result, combined with potential insulin resistance (10), suggests that free fatty acid activation of PPAR $\alpha$  can initiate PDK4 gene expression near the onset of hibernation despite relatively high serum insulin levels.

Another activity of PPAR $\alpha$  can be seen in the activation of genes involved in lipid metabolism (26). Of key importance to hibernation is the potential role of PPAR $\alpha$  in coordinating PDK4 gene expression with the expression of genes responsible for extracellular and intracellular lipid transport and mitochondrial  $\beta$ -oxidation of fatty acids (reviewed in Ref. 27). As shown in Fig. 6, activation of PPAR $\alpha$  provides a mechanism linking the inhibition of carbohydrate oxidation with increased fatty acid oxidation, thus accounting for the switch in fuel selection during hibernation. PPAR $\alpha$  activity has also been associated with expression of uncoupling protein-3 (UCP3; Ref. 7). This finding connecting PPAR $\alpha$  to UCP gene expression is especially relevant with respect to hibernation and nonshivering thermogenesis (5). In consideration of its role in the activation of genes associated with carbohydrate utilization, lipid catabolism, and nonshivering thermogenesis, PPAR $\alpha$  is likely to act as a common regulator of the metabolic response during hibernation.

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The new sequence reported in this paper has been deposited in the GenBank database (accession no. AY038604).

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## REFERENCES

1. **Andrews MT, Squire TL, Bowen CM, and Rollins MB.** Low-temperature carbon utilization is regulated by novel gene activity in the heart of a hibernating mammal. *Proc Natl Acad Sci USA* 95: 8392–8397, 1998.
2. **Bauer VW, Squire TL, Lowe ME, and Andrews MT.** Expression of a chimeric retroviral-lipase mRNA confers enhanced lipolysis in a hibernating mammal. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R1186–R1192, 2001.
3. **Bauman WA.** Seasonal changes in pancreatic insulin and glucagon in the little brown bat (*Myotis lucifugus*). *Pancreas* 5: 342–346, 1990.
4. **Bowker-Kinley MM, Davis WI, Wu P, Harris RA, and Popov KM.** Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J* 329: 191–196, 1998.
5. **Boyer BB, Barnes BM, Lowell BB, and Grujic D.** Differential regulation of uncoupling protein gene homologues in multi-

- ple tissues of hibernating ground squirrels. *Am J Physiol Regulatory Integrative Comp Physiol* 275: R1232–R1238, 1998.
6. **Brooks SPJ and Storey KB.** Mechanisms of glycolytic control during hibernation in the ground squirrel *Spermophilus lateralis*. *J Comp Physiol [B]* 162: 23–28, 1992.
  7. **Brun S, Carmona MC, Mampel T, Vinas O, Giralt M, Iglesias R, and Villarroya F.** Activators of peroxisome proliferator-activated receptor- $\alpha$  induce the expression of the uncoupling protein-3 gene in skeletal muscle: a potential mechanism for the lipid intake-dependent activation of uncoupling protein-3 gene expression at birth. *Diabetes* 48: 1217–1222, 1999.
  8. **Feinberg AP and Vogelstein B.** A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6–13, 1983.
  9. **Feinberg AP and Vogelstein B.** A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity (Addendum). *Anal Biochem* 137: 266–267, 1984.
  10. **Florant GL, Lawrence AK, Williams K, and Bauman WA.** Seasonal changes in pancreatic B-cell function in euthermic yellow-bellied marmots. *Am J Physiol Regulatory Integrative Comp Physiol* 249: R159–R165, 1985.
  11. **Florant GL, Nuttle LC, Mullinex DE, and Rintoul DA.** Plasma and white adipose tissue lipid composition in marmots. *Am J Physiol Regulatory Integrative Comp Physiol* 258: R1123–R1131, 1990.
  12. **Forman BM, Chen J, and Evans RM.** Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors  $\alpha$  and  $\delta$ . *Proc Natl Acad Sci USA* 94: 4312–4317, 1997.
  13. **Galster WA and Morrison P.** Seasonal changes in serum lipids and proteins in the 13-lined ground squirrel. *Comp Biochem Physiol* 18: 489–501, 1966.
  14. **Garfinkel AS and Schotz MC.** Separation of molecular species of lipoprotein lipase from adipose tissue. *J Lipid Res* 13: 63–68, 1972.
  15. **Harris RA, Huang B, and Wu P.** Control of pyruvate dehydrogenase kinase gene expression. *Adv Enzyme Regul* 41: 269–288, 2001.
  16. **Heldmaier G, Klingenspor M, Werneker M, Lampi BJ, Brooks SP, and Storey KB.** Metabolic adjustments during daily torpor in the Djungarian hamster. *Am J Physiol Endocrinol Metab* 276: E896–E906, 1999.
  17. **Hutson NJ, Kerbey AL, Randle PJ, and Sugden PH.** Conversion of inactive (phosphorylated) pyruvate dehydrogenase complex into active complex by the phosphatase reaction in heart mitochondria is inhibited by alloxan-diabetes or starvation in the rat. *Biochem J* 173: 669–680, 1978.
  18. **Kliwer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, and Lehmann JM.** Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$ . *Proc Natl Acad Sci USA* 94: 4318–4323, 1997.
  19. **Kliwer SA, Umesono K, Noonan DJ, Heyman RA, and Evans RM.** Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* 358: 771–774, 1992.
  20. **Lyman CP, Willis JS, Malan A, and Wang LCH.** *Hibernation and Torpor in Mammals and Birds*. New York, NY: Academic Press, 1982.
  21. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, and Evans RM.** The nuclear receptor superfamily: the second decade. *Cell* 83: 835–839, 1995.
  22. **Pengelly ET and Fisher KC.** Rhythmical arousal from hibernation in the gold-mantled ground squirrel, *Citellus lateralis tescorum*. *Can J Zool* 39: 105–120, 1961.
  23. **Randle PJ.** Fuel selection in animals. *Biochem Soc Trans* 14: 799–806, 1986.
  24. **Rowles J, Scherer SW, Xi T, Majer M, Nickle DC, Rommens JM, Popov KM, Harris RA, Riebow NL, Xia J, Tsui LC, Bogardus C, and Prochazka M.** Cloning and characterization of PDK4 on 7q21.3 encoding a fourth pyruvate dehydrogenase kinase isoenzyme in human. *J Biol Chem* 271: 22376–22382, 1996.
  25. **Sambrook J, Fritsch EF, and Maniatis T.** *Molecular Cloning*. New York: Cold Spring Harbor Laboratory Press, 1989.
  26. **Schoonjans K, Staels B, and Auwerx J.** The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochim Biophys Acta* 1302: 93–109, 1996.
  27. **Squire TL and Andrews MT.** Genetic control of carbon utilization during hibernation: mechanistic considerations. In: *Life in the Cold: Eleventh International Hibernation Symposium*, edited by Heldmaier G and Klingenspor M. Berlin: Springer-Verlag, 2000, p. 325–337.
  28. **Tokuyama K, Galantino HL, Green R, and Florant GL.** Seasonal glucose uptake in marmots (*Marmota flaviventris*): the role of pancreatic hormones. *Comp Biochem Physiol A Physiol* 100: 925–930, 1991.
  29. **Wang LCH.** Time patterns and metabolic rates of natural torpor in the Richardson's ground squirrel. *Can J Zool* 57: 149–155, 1979.
  30. **Wu P, Blair PV, Sato J, Jaskiewicz J, Popov KM, and Harris RA.** Starvation increases the amount of pyruvate dehydrogenase kinase in several mammalian tissues. *Arch Biochem Biophys* 381: 1–7, 2000.
  31. **Wu P, Inskeep K, Bowker-Kinley MM, Popov KM, and Harris RA.** Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. *Diabetes* 48: 1593–1599, 1999.
  32. **Wu P, Peters JM, and Harris RA.** Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor  $\alpha$ . *Biochem Biophys Res Commun* 287: 391–396, 2001.
  33. **Wu P, Sato J, Zhao Y, Jaskiewicz J, Popov KM, and Harris RA.** Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. *Biochem J* 329: 197–201, 1998.